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PRINCIPAL INVESTIGATOR: Michael H. Nantz

CONTRACTING ORGANIZATION: University of Louisville Research Foundation
Louisville, KY 40245

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Michael H. Nantz
Email: Michael.nantz@louisville.edu

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14. ABSTRACT

We report synthetic routes for modifying the fluorophore cypate, an indocyanine green (FDA-approved) derivative, with custom thiol-terminated peptide and poly(ethylene glycol) (PEG) substrates. We selected the amino acid sequence of the peptide to serve as a recognition element for the enzyme urokinase plasminogen activator (uPA), a serine protease synthesized within cancer cells. In collaboration with Dr. Kang (partnering award W81XWH-08-1-0460), the peptide and PEG conjugates are attached via the thiol functionality to nanogold particles for evaluation of their fluorescence properties. We also describe the synthesis of a novel cypate derivative that possesses two pendant aldehyde groups. This derivative will be useful for performing straightforward coupling reactions with aminooxy substrates via oximation (oxime ether formation). In this way, we aim to conduct the loading of cypate onto nanogold particles fitted with aminooxy-functionalized peptide or PEG fragments by simple mixing in water.

15. SUBJECT TERMS
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Introduction

We seek to prepare a fluorescing nano-entity that can be effectively used for cancer detection and diagnosis. Our goal is to functionalize nanogold particles with the fluorescent probe cypate and a cancer enzyme recognition element. Attachment of these two components onto nanogold particles will be engineered such that the fluorescence emission of cypate is quenched until the nano-entity encounters a cancer-specific enzyme. Encounter with the enzyme, selected as uPA for this study, will result in a peptide bond cleavage to release constrained cypate so that fluorescence emission can occur and serve as a signal. To accomplish this goal, we propose to attach cypate to a nanogold particle using two spacers, a short peptide-based spacer containing a uPA recognition element and a longer, enzyme-resistant poly(ethylene glycol) (PEG) based spacer. As long as cypate is tethered to the nanogold particle surface via the short spacer, its fluorescence emission will be quenched due to the influence of the surface plasmon polarion field of the nanometal. However, after the short spacer is selectively cleaved by uPA, cypate is free to migrate to the length of the long spacer. Migration of the cypate away from the surface of the nanometal not only results in fluorescence emission dequenching but also may result in enhanced fluorescence if the distance of the long spacer is appropriately controlled. The research thus seeks to secure synthetic routes to peptide- and PEG-conjugates of cypate, to develop methods for their attachment to nanogold particles, and to optimize the fluorescence properties of the nano-entity as well as its interactions with uPA.

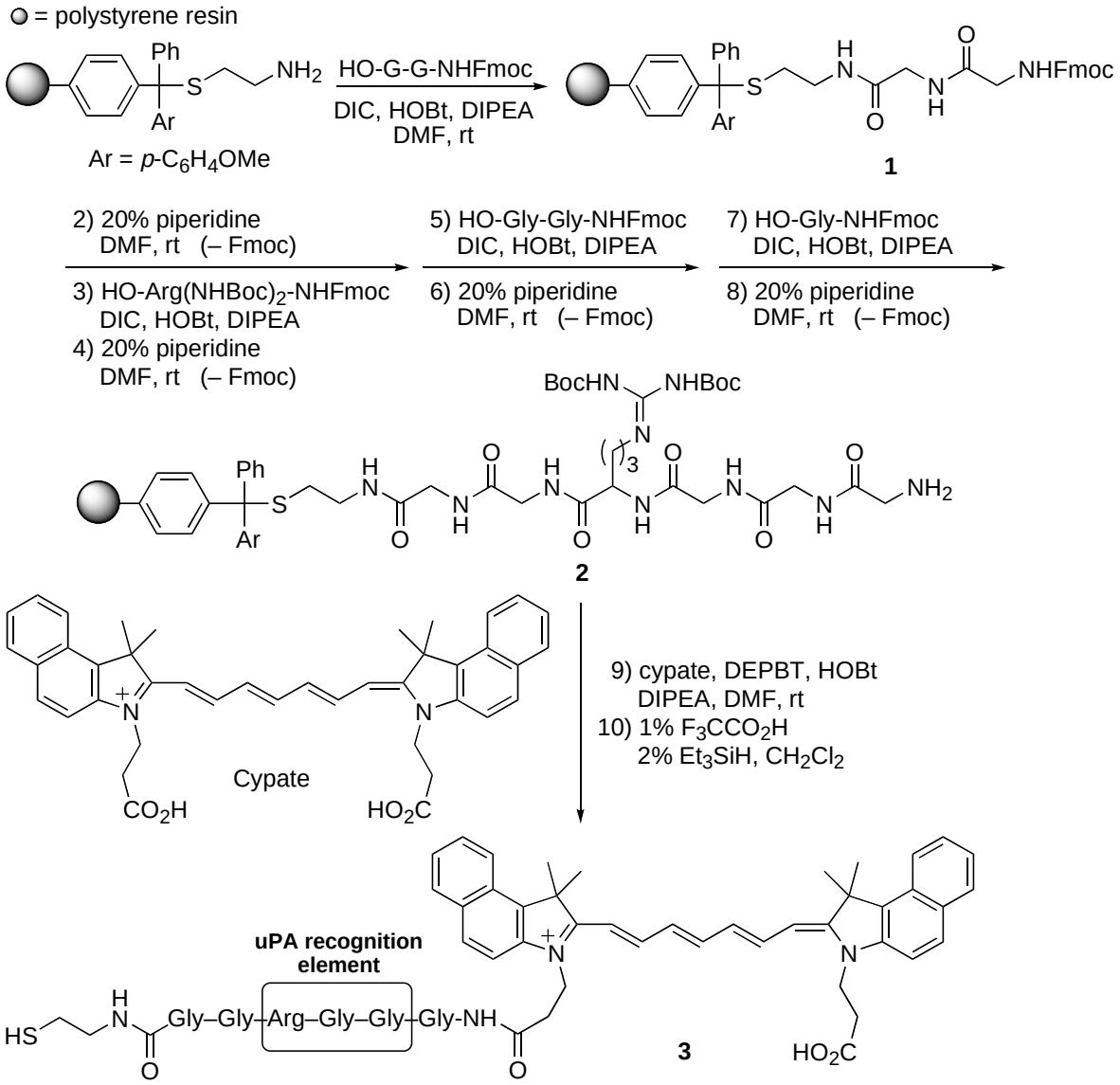
Body – Summary of Synthetic Efforts

A. Synthesis of a prototype peptide-cypate conjugate (the 'short spacer')

General considerations. As indicated in our Statement of Work and the Introduction above, we aimed to prepare a peptide-cypate conjugate that (a) can be attached to the surface of a nanogold particle (NGP), (b) is of an appropriate length so that cypate fluorescence emission is quenched by the resident gold surface plasmon field, and (c) can be cleaved by the enzyme urokinase plasminogen activator (uPA), a serine protease synthesized within cancer cells. With these goals in mind, we selected as our target conjugate the thiol-functionalized, peptide-cypate conjugate **3** (Scheme 1). The specific amino acid sequence in this prototype follows from the literature that the Gly-Gly-Arg motif is selectively cleaved by uPA (Zimmerman 1978, Chung, 2006) and the length of the peptide chain (6 amino acids plus cysteamine) was chosen based on initial observations by Kang *et al.* (Kang and Hong, 2006) and subsequent observations by our collaborator Achilefu *et al.* (Achilefu, 2008), who showed that cypate fluorescence emission was quenched when cypate was attached to a NGP via an 8 amino acid spacer.

Synthesis. Methoxy-activated, cysteamine-loaded polystyrene resin (Novabiochem) was reacted with the Fmoc-protected amino acids depicted in Scheme 1 following the standard protocol of DIC-mediated coupling and piperidine-mediated deprotection to obtain the resin-bound Boc-protected peptide **2**. The peptide chain in **2** was cleaved from the resin (TFA) and analyzed by HPLC to confirm homogeneity. Several coupling procedures were examined to optimize attachment of cypate to the N-terminus of the peptide chain of **2** to minimize the number of cypate equivalents consumed. While polymer site isolation effectively prevented reaction at both carboxylic acid groups of cypate, an excess of cypate (*ca.* 4 equiv.) was required to achieve modest coupling yields. This result is in agreement with previous reports on synthesis of peptide-cypate conjugates (Fan, 2007; Ye, 2006). We determined that the cypate coupling using DEPBT (Ye, 2005) was most effective (A. Massey, unpublished). Cleavage of the conjugate from the resin was accomplished using minimal TFA in the presence of triethylsilane. Standard cleavage conditions using 50% TFA resulted in cypate decomposition.

In general, based on the theoretical concentration of resin reactive sites, conjugate **3** was obtained in 8-12% yield (based on total cypate consumed; cleavage yields 60-62%). HPLC purification of **3** also exacted a toll on material in that the conjugate appears sensitive to TFA present in the eluent.



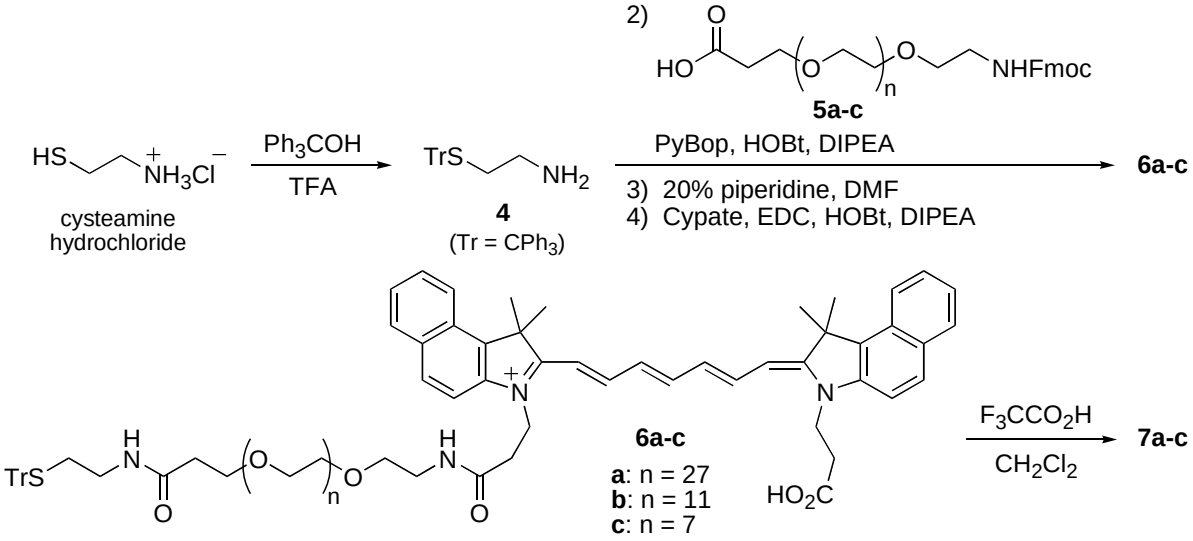
Scheme 1. Solid phase synthesis of peptide-cypate conjugate.

Improvement. While this approach delivered sufficient material to test the validity of cypate quenching (i.e., test short spacer length) as well as to probe whether uPA-mediated peptide cleavage can occur in the proximity of the NGP surface (see Initiating PI report, award number W81XWH-08-1-0460), we believe the design of a route to circumvent hydrophilic peptide (or PEG) conjugate synthesis and purification would constitute a much needed improvement in the experimental design. Along these lines, we plan to develop and test an oximation strategy for future cypate-related couplings (see sections C & D below).

B. Synthesis of poly(ethylene glycol)-cypate conjugates (the 'long spacer')

General considerations. As originally proposed, we selected poly(ethylene glycol) (PEG) to comprise the long spacer. PEG is resistant to enzymatic cleavage, a key design feature. Our synthetic goal was to covalently attach thiol-modified (for adsorption to NGP) PEG-based spacers to cypate. We have developed a solution phase synthesis of our target, thiol-PEG conjugate **7** (Scheme 2); however, we foresee adaptation of this route to the solid phase in future iterations. Our initial assumption was that PEG spacers providing 'distance-from-NGP surface' of up to 10 nm would be required to adequately position cypate to enhance its fluorescence emission. On synthesis and evaluation of the first prototypes, 'PEG-27' analog **7a** and 'PEG-11' analog **7b**, revealed that the shorter analog **7b** appeared to better position cypate. Consequently, our subsequent focus was, and continues to be, in the shorter range (i.e., PEG or PEG-like linear distance similar to **6b**).

Synthesis. Cysteamine hydrochloride was trityl protected and the resultant amine **4** was coupled to commercial PEG reagents **5a** (n = 27, Novabiochem), **5b** (n = 11, Novabiochem) and **5c** (n = 7, Fluka) using PyBop as the coupling agent. Although the conjugates were quite polar, we found that purification proceeded best under normal phase (silica gel) conditions. Standard Fmoc removal was followed by EDC-mediated coupling to cypate, giving cypate conjugates **6a-c.** These conjugates also were purified by silica gel column chromatography. HRMS analyses were performed on the trityl-protected conjugates to avoid complications arising from disulfide formation. The trityl group was cleaved prior to studies involving loading onto NGP.



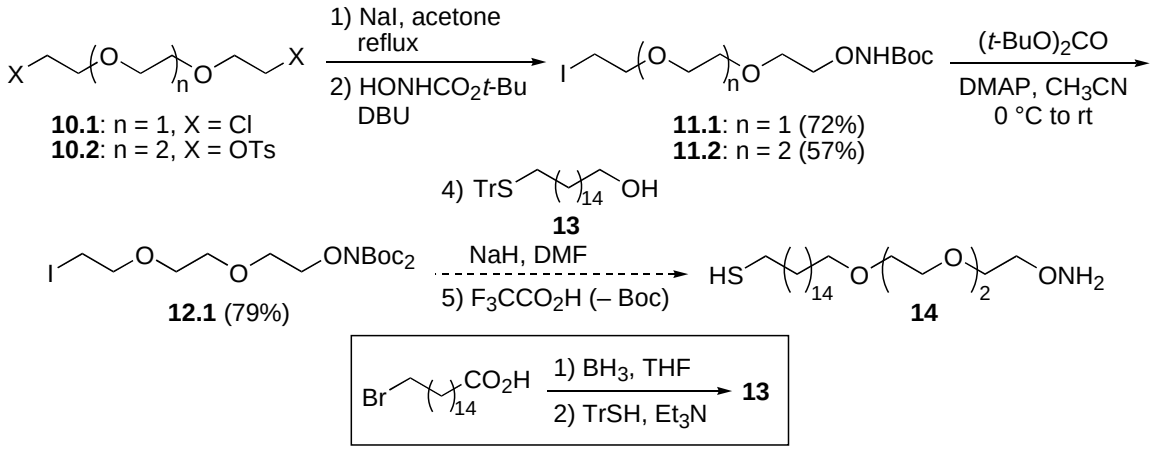
Scheme 2. Solution phase synthesis of PEG-cypate conjugates.

Problems with solution phase synthesis. Cypate coupling to the TrS-PEG-NH_2 reagents **5a**-**c** (step 4, Scheme 2) gives mono-coupled materials **6a**-**c** as well as some bis-coupled material (i.e., both carboxylic acid sidechains of cypate react). We verified formation of a bis-coupled material during synthesis of **6b**. HRMS analysis of a major side product confirmed that two PEG_{11} chains had been attached to cypate ($C_{137}H_{190}N_6O_{29}S_2$ $[M+2H]^+$, calcd 1225.1598, found 1225.1305). We do not expect this competitive reaction to be a problem during the synthesis of our proposed, optimal peptide-cypate-PEG target since attachment of the PEG (long chain) unit will occur while cypate is attached to the peptide chain anchored on solid phase.

C. Synthesis of an aminooxy-terminated amphiphilic thiol for nanoparticle targeting.

General considerations. As outlined in our research plan, we aimed to develop an aminooxy approach for the attachment of cancer specific ligands to the cypate-modified NGPs. This strategy requires the synthesis of a thiol-PEG-ONH_2 reagent that effectively binds to NGPs and presents the aminooxy group (ONH_2) at the surface for chemospecific ligation to aldehyde- or ketone-functionalized ligands. We have now designed this spacer to contain a significant hydrophobic domain proximal to the thiol moiety to facilitate assembly on the gold surface (monolayer formation). Specifically, as illustrated by compound **14** (Scheme 3) the general design is HS-hydrophobic-hydrophilic-ONH_2. Furthermore, we believe that the ease of oximation (aminooxy plus aldehyde or ketone) in water suggests using this chemistry to couple cypate, either to the peptide fragments or PEG chains. Consequently, we have secured a synthesis of the hydrophilic component of our spacer and are now pursuing completion of the hydrophobic domain.

Synthesis. Reaction of the diiodide formed from commercial PEG reagents **10** with Boc-protected hydroxylamine gave iodo-PEG reagents **11** in good yield (Scheme 3). We have found that treatment of **11.1** with nucleophiles under basic conditions surprisingly (10 membered ring) gives the cyclized product arising from intramolecular iodide displacement by the deprotonated Boc-protected aminooxy group (presumably a consequence of metal-assisted template formation via a crown ether-like transition state). Consequently, this necessitated the addition of a second Boc protecting group. Bis-(Boc-protected) analog **12.1** has been prepared and is now available for reaction with the alcoholate derived from hydrophobic alcohol **13.** Thiol spacer **14** is presently unknown. Reagent **13** has been described in the literature (Hornillos, 2006).



Scheme 3. Synthesis of aminooxy thiol spacer.

D. Synthesis of a cypate-aldehyde.

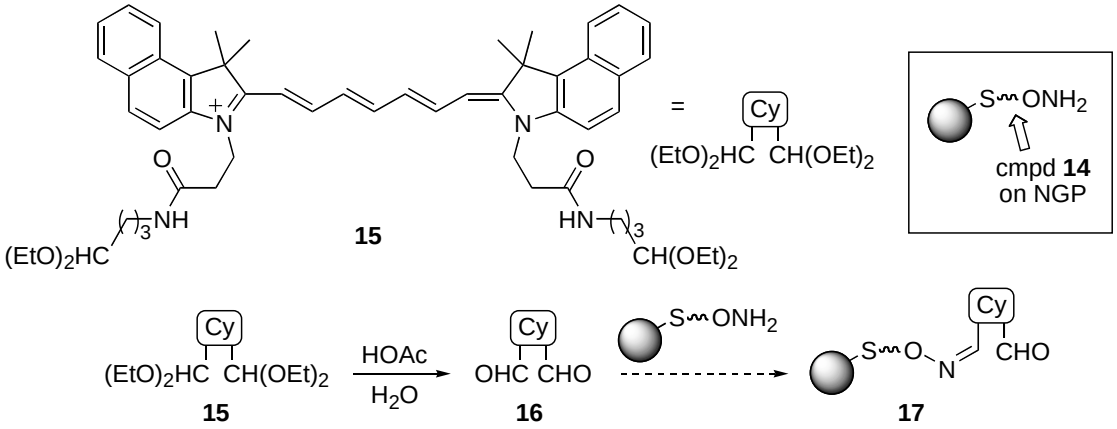
To enable the direct labeling of NGP–ONH_2 (e.g., NGP treated with thiol reagent **14**) with cypate using the oximation strategy, we required cypate fitted with aldehyde functionality. Our initial efforts to prepare either (aldehyde)-cypate-(carboxylic acid) or $(aldehyde)_2$cypate focused on direct transformation of the carboxylic acid groups of cypate to the corresponding mono- or bis-aldehyde using the following approaches:

(1) $–CO_2H \rightarrow –CH_2OH$, then selective oxidation to –CHO. Result: all reduction attempts (BH_3, THF) failed, no cypate diol isolated;

(2) $-CO_2H \rightarrow -C(O)Cl$, then selective reduction to –CHO. Result: acid chloride successfully prepared (oxalyl chloride); however, all attempts to reduce the derived acid chloride ($LiAlH(O\textit{t}\text{-}Bu)_3$, Dibal-H) failed.

We next turned our attention to modifying cypate via bis-amide formation using a reagent that contains masked aldehyde functionality. Using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to mediate the dehydration (HOBt, DIPEA, CH_2Cl_2, 0 °C to rt), cypate was successfully coupled to the amino acetal $H_2N(CH_2)_3CH(OEt)_2$ (Aldrich) to afford cypate amide-acetal **15** (Scheme 4) (Biswas, unpublished results). No mono-amide product was isolated; however, the reaction has not yet been fully explored. We expect that, if needed, conditions can be developed to obtain the mono-derivative (amide acetal)-cypate-(CO_2H) as a major product. Amide-acetal **15** has been characterized by ^1H NMR and HRMS ($C_{57}H_{75}N_4O_6^+$ calcd 911.5681, found 911.5681). On pilot scale (4 mg), the treatment of **15** with aq. acetic acid at room temperature effected hydrolysis of the acetal moieties to give the corresponding bis-aldehyde analog **16**.

At this stage, we envision that suitably aminooxy-functionalized nanogold particles can be directly reacted in water with bis-aldehyde derivative **16** (or its mono-amide analog) to result in cypate attachment via oxime ether formation (e.g., **16** → **17**). This approach can be adapted to circumvent synthesis of either PEG-cypate or peptide-cypate conjugates prior to NGP attachment, thus avoiding the accompanying purification difficulties. Efforts to fully exploit this strategy will follow synthesis of the prototype thiol spacer **14** (Scheme 3).



Scheme 4. Oximation approach for attaching cypate to NGP.

E. Future synthesis efforts

We are poised to attach cypate to NGPs via aminooxy ligation chemistry in that aldehyde-modified cypate and aminooxy thiol spacers are at hand. As the lengths for the short and long spacers are near confirmation, we expect to prepare the dual chain motif as outlined in the Statement of Work using the new oximation coupling technology. Having prepared peptide-cypate, PEG-cypate, and aldehyde-cypate conjugates, we are intimately familiar with the chemistry needed to complete synthesis of a functional NGP preparation for testing in cancer cells.

Key Research Accomplishments

- Secured syntheses of peptide–cypate conjugates fitted with thiol functionality

  Key observation (from partnering award W81XWH-08-1-0460): the overall length of the peptide spacer in peptide-cypate conjugate **3** (Scheme 1) is suitable in that 81-86% of cypate fluorescence is quenched relative to controls when loaded onto 5 nm gold particles.

- Secured syntheses of PEG–cypate conjugates fitted with thiol functionality

  Key observation (from partnering award W81XWH-08-1-0460): fluorescence enhancement greater than 10% was noted, although not optimized, only at lengths shorter than the overall length of the HS-PEG_{27}-cypate conjugate, calculated to be ~10 nm. This obviates syntheses of longer spacers for attachment to nanogold particles ≤ 10 nm in size.

- Prepared a cypate–bis(aldehyde) substrate for use in conjugation (loading) reactions

  The availability of compound **16** is expected to facilitate the attachment of cypate to aminooxypeptide- or aminooxyPEG-based spacers that already have been positioned on nanogold particles. This strategy is expected to be more efficient (and economical) than the loading of fully formed peptide-cypate or peptide-PEG conjugates.

Reportable Outcomes

This award is partnered with award W81XWH-08-1-0460 (Initiating PI: Kang). The co-deliverables are listed below (documents not attached as part of this report).

- Manuscript

  J Wang, MH Nantz, S Achilefu and KA Kang. "FRET-Like Fluorophore-Nanoparticle Complex for Highly Specific Cancer Localization." ISOTT 2008 Conference Proceedings, *Advances in Experimental Medicine and Biology*, in press.

- Abstract

  Jianting Wang,[1] Martin O'Toole,[1] Archna P. Massey,[2] Souvik Biswas,[2] Michael H. Nantz,[2] Samuel Achilefu,[3] and Kyung A. Kang.[1] "Highly specific, NIR fluorescent contrast agent with emission controlled by gold nanoparticle." To be presented at the 37th Annual ISOTT Conference, Cleveland, OH; July 5-9, 2009.

  Departments of [1]Chemical Engineering, [2]Chemistry, University of Louisville, Louisville, KY 40292, USA; [3]Department of Radiology, Washington University School of Medicine, St. Louis, MO 63110, USA

- Patent

  Kang, K.; Nantz, M. H. Site Specific Fluorescence Marking and Contrast Marker for Same. PCT Application No. PCT/US2009/039025; filed March 31, 2009 (U*of*L OTT 07074).

  STATEMENT OF GOVERNMENT INTEREST. This invention was made with government support under DOD Grant No. W81XWH-08-1-0460 awarded by Department of Defense (DOD). The government has certain rights in the invention.

  FIELD OF THE INVENTION. A field of the invention is in vivo imaging and cell detection via fluorescent markers. Example applications of the invention include fluorescence detection of breast cancer and other cancers with a site specific enhanced fluorescence marker.

- Degrees

  This award has provided funds to train Mr. Souvik Biswas as a Graduate Student Researcher during his third year of graduate school (U*of*L, Ph.D. program in Chemistry).

Conclusion

We have developed the synthetic routes necessary to prepare thiol terminated peptide- and PEG-based conjugates of cypate, an indocyanine green (FDA-approved) derivative with two pendant carboxylic acid groups developed by our collaborator Dr. S. Achilefu. Examples of both types of derivatives have been prepared and evaluated by our collaborator, Dr. K. Kang, as part of the joint effort to optimize the fluorescence properties of the proposed nano-entity. A key aspect of the conjugation research involves attaching peptide- or PEG-based spacers to the cypate carboxylic acid groups via amide bond formation. We are now moving toward using an oximation approach (aldehyde + aminooxy) for these conjugations. Along these lines, we have successfully prepared in good yield a novel derivative of cypate that has two pendant aldehyde groups. The facile ligation reaction *in water* between aldehydes (RCHO) and aminooxy groups ($R'ONH_2$) yielding robust oxime ethers (RC=NOR') suggests that our new cypate bis-aldehyde can be used to directly modify nanogold particles that have been previously fitted with aminooxy peptide or aminooxy PEG spacers. The efficiency and flexibility of this approach is expected to improve the loading of cypate onto nanogold particles and thus benefit the optimization process.

The technology described in this report enables the dual modification of cypate with a uPA selective peptide spacer and a longer PEG spacer such that the doubly functionalized conjugate can be loaded onto nanogold particles. Cypate fluorescence will remain quenched until the nano-entity encounters uPA (cancer marker). In collaboration with Dr. Kang, we are now seeking to optimize recognition of the peptide sequence by uPA so that peptide cleavage occurs to allow cypate to migrate away from the nanogold surface and fluoresce. The intact PEG spacer would not only ensure that cypate remains attached to the particle, but also may enhance cypate fluorescence emission if appropriately positioned from the nanogold surface. This is a crucial variable that is also under investigation. With the remaining variables optimized, such a nano-entity would constitute a highly specific fluorescing cancer locator.

References

Achilefu, S.; Ye, Y.; Zhang, Z.; Lee, H.; Fan, J.; Liang, K.; Berezin, M.; Cheney, P.; Edwards, W. B. Chemical and biochemical contrast strategies for optical imaging of molecular processes in vivo. Abstracts of Papers, 236th ACS National Meeting, Philadelphia, PA, United States, August 17-21, 2008 (2008).

Chung, D.-E.; Kratz, F. Development of a novel albumin-binding prodrug that is cleaved by urokinase-type-plasminogen activator (uPA). *Biorg. & Med. Chem. Lett.* **2006**, *16*, 5157-5163.

Hornillos, V.; Saugar, J. M.; de la Torre, B. G.; Andreu, D.; Rivas, L.; Acuna, A. U.; Amat-Guerri, F. Synthesis of 16 mercaptohexadecylphosphocholine, a miltefosine analog with leishmanicidal activity. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5190-5193.

Ye, Y.; Bloch, S.; Xu, B.; Achilefu, S. Design, synthesis, and evaluation of near infrared fluorescent multimeric RGD peptides for targeting tumors. *J. Med. Chem.* **2006**, *49*, 2268-75.

Ye, Y.; Bloch, S.; Kao, J.; Achilefu, S. Multivalent Carbocyanine Molecular Probes: Synthesis and Applications. *Bioconjugate Chem.* **2005**, *16*, 51-61.

Ye, Y.; Li, H.; Jiang, X. DEPBT as an efficient coupling reagent for amide bond formation with remarkable resistance to racemization. *Peptide Sci.* **2005**, *80*, 172-178.

Zimmerman, M.; Quigley, J. P.; Ashe, B.; Dorn, C.; Goldfarb, R.; Troll, W. Direct fluorescent assay of urokinase and plasminogen activators of normal and malignant cells: kinetics and inhibitor profiles. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 750-753.

Appendices

A. Experimental Procedures

1. General

Solid phase peptide synthesis was performed using methoxy-activated, cysteamine-loaded polystyrene resin (Novabiochem, cat. no. 01-64-0086). All amino acids (Novabiochem) and peptide coupling reagents (Aldrich Chemical Company) were used as received from vendors. DMF (Aldrich) was used as received, and CH_3CN (Pharmaco) was filtered and degassed (N_2) prior to use. HPLC analyses and purifications were conducted on a Waters Corporation Delta 600 instrument. High resolution mass spectrometry services were provided by the Center for Regulatory and Environmental Analytical Metabolomics at the University of Louisville; FTCIR measurements were taken on a Thermo LTQ-FT instrument (7 tesla). Cypate was provided by our collaborator Dr. Samuel Achilefu (Washington University, St. Louis).[1]

2. Solid phase synthesis of conjugate 3 (steps according to Scheme 1)

Steps 1&2. Cysteamine-loaded polystyrene resin (200 mg, 0.14 mmol active amine) was treated with Fmoc-protected diglycine (Novabiochem, 150 mg, 0.42 mmol) in the presence of diisopropylcarbodiimide (DIC, 0.064 mL, 0.42 mmol), *N*-hydroxybenzotriazole (HOBt, 56 mg, 0.42 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 0.12 mL, 0.84 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH_2Cl_2 (7X). The resin then was dried under vacuum, swelled using DMF, filtered, and treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH_2Cl_2 (5X), and then dried under vacuum.

Steps 3&4. The diglycine loaded resin (®-G-G-NH_2) was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected arginine-Boc_2 (Novabiochem, 250 mg, 0.42 mmol), DIC (0.064 mL, 0.42 mmol), HOBt (56 mg, 0.42 mmol) and DIPEA (0.12 mL, 0.84 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH_2Cl_2 (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH_2Cl_2 (5X), and then dried under vacuum.

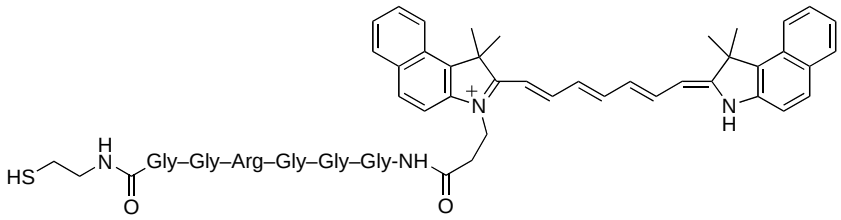
Steps 5&6. The ®-G-G-R-NH_2 resin was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected diglycine (Novabiochem, 250 mg, 0.70 mmol), DIC (0.108 mL, 0.70 mmol), HOBt (90 mg, 0.70 mmol) and DIPEA (0.25 mL, 1.4 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH_2Cl_2 (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH_2Cl_2 (5X), and then dried under vacuum.

Steps 7&8. The ®-G-G-R-G-G-NH_2 resin was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected glycine (Novabiochem, 205 mg, 0.70 mmol), DIC (0.108 mL, 0.70 mmol), HOBt (90 mg, 0.70 mmol) and DIPEA (0.25 mL, 1.4 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH_2Cl_2 (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH_2Cl_2 (5X), and then dried under vacuum.

To confirm peptide integrity prior to coupling with cypate, a small portion of the peptide was cleaved from the resin by shaking the ®-G-G-R-G-G-G-NH_2 resin in a 1:10:10 mixture of triethylsilane:TFA:CH_2Cl_2 at room temperature for 4h. The reaction was filtered and solvents removed under reduced pressure. The residue was washed with Et_2O (7X) and then dried under vacuum. Analysis of the crude cysteamine-hexapeptide conjugate by HPLC (Atlantis dC18 5 mm column, gradient elution using 5% CH_3CN/H_2O to 95% CH_3CN/H_2O over 30 min) indicated excellent sample homogeneity (retention time = 25.6 min); HRMS (FTCIR): $C_{33}H_{45}N_{10}O_8S$ $[M+H]^+$, calcd 741.3137, found 741.3133.

Step 9. To the ®-G-G-R-G-G-G-NH_2 resin (65 mg, theoretical 0.045 mmol peptide) in DMF at room temperature was added cypate (140 mg, 0.227 mmol), 3-(diethoxyphosphoryloxy)-1, 2, 3-benzotriazin-4(3H)-one (DEPBT, Aldrich, 68 mg, 0.227 mmol), HOBt (30 mg, 0.227 mmol), and DIPEA (0.08 mL, 0.45 mmol). After shaking overnight, the resin was filtered and thoroughly washed with CH_2Cl_2 (7X). The resin then was dried under vacuum.

Step 10. To the ®-G-G-R-G-G-G-NHC(O)-cypate resin prepared above (15 mg, theoretical 0.01 mmol) in CH_2Cl_2 (0.3 mL) at room temperature was added triethylsilane (0.06 mL). After shaking 15 minutes, trifluoroacetic acid (0.04 mL) was added and the resin suspension was shaken 1 h, allowed to stand 1 h, and then shaken 3 h. The resin was filtered and the filtrate was concentrated by rotary evaporation. The residue was washed with Et_2O (5X) and dried under vacuum to afford the target cysteamine-hexapeptide-cypate conjugate (7 mg, 62% based on resin loading, 12% based on cypate consumption); HRMS (FTCIR): $C_{59}H_{73}N_{12}O_9S$ $[M]^+$, calcd 1125.5338, found 1053.5114 (loss of acrylic acid $[M^+ – HO_2CCH=CH_2]$ as shown below, calcd for $C_{56}H_{69}N_{12}O_7S$, 1053.5127, see Appendix B2 for spectrum).



3. Solution phase synthesis of TrS-PEG-cypate 6b (steps according to Scheme 2)

Step 2. To PEG-reagent **5b** (110 mg, 0.13 mmol) and trityl-protected cysteamine **4** (42 mg, 0.13 mmol) in CH_2Cl_2 (5 mL) at room temperature was added HOBt (18 mg, 0.13 mmol) and DMF (0.2 mL). The reaction mixture was cooled to 0 °C and DIPEA (0.56 mL, 0.33 mmol) was added followed by addition of a solution of PyBop (68 mg, 0.13 mmol) in CH_2Cl_2 (2 mL). The mixture was gradually warmed to room temperature and stirred overnight. The reaction solvents were removed under high vacuum. The solid residue was dissolved in EtOAc and the solution was washed successively with water, then brine and subsequently dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation and the crude product purified by column chromatography (SiO_2, 2% MeOH in CH_2Cl_2) to afford the amide product (109 mg).

Step 3. The TrS-PEG-NHFmoc product (104 mg, 0.091 mmol) was treated with piperidine (0.168 mL) in DMF (2 mL) at room temperature. After stirring 4h, the DMF was removed under high vacuum. The residue was washed with Et_2O (5X) and dried under vacuum to give the deprotected product (63 mg, 79%). HRMS, calc'd $C_{48}H_{74}O_{13}N_2S$ 919.4984, found 919.4995.

Step 4. To a suspension of the TrS-PEG-NH_2 product (52 mg, 0.056 mmol), HOBt (8 mg, 0.059 mmol) and cypate (35 mg, 0.056 mmol) in CH_2Cl_2 (3 mL) at room temperature was added DMF (0.2 mL). The reaction mixture was cooled to 0 °C and DIPEA (22 mg, 0.17 mmol) was added followed by addition of a solution mixture of EDC (9 mg, 0.47 mmol) in CH_2Cl_2 and DMF. After stirring in the dark at room temperature overnight, the reaction was quenched by addition

of water and extracted using CH_2Cl_2. The organic layer was washed with brine and then dried (Na_2SO_4). The solvents were removed by rotary evaporation and the residue purified by column chromatography (SiO_2, gradient elution 9-15% MeOH in CH_2Cl_2) to obtain mono-coupled product **6b** (7 mg, 9%) [HRMS, $C_{89}H_{113}N_4NaO_{16}S$ [M+Na^+], calcd 1548.7770, found 1543.8318] and bis-coupled product (12 mg, 10%) [HRMS, $C_{137}H_{190}N_6O_{29}S_2$ [M+2H]$^+$, calcd 1225.1598, found 1225.1305].

4. Synthesis of heterobifunctional PEG spacer 12.1 (steps according to Scheme 3)

Step 1. 16.02 g of NaI (106.9 mmol), 10.0 g (53.45 mmol) of 1,2-bis(2-chloro-ethoxy)ethane were dissolved in 110 mL of acetone and fitted the flask with a reflux condensor. The reaction mixture was refluxed for 20 h, cooled to room temperature, removed the acetone by vacuum, and added 150 mL of chloroform. Filtered the precipitate using frit funnel, removed the filtrate under vacuum to obtain yellow oily compound. The crude mixture was subjected to silica gel chromatography to give 18.5 g (94%) of the corresponding diiodide; ^{1}H NMR ($CDCl_3$): δ 3.78-3.69 (m, 4H), 3.68-3.58 (m, 4H), 3.25-3.19 (m, 4H).

Step 2. 3.6 g (9.72 mmol) of I-PEG_3-I, 323 mg (2.43 mmol) of HO-NH(Boc) were taken in a flask, stirred to obtain a clear solution, and added 0.363 mL (2.43 mmol) of DBU dropwise. The reaction mixture was stirred 1hr (during this time reaction mixture solidifies). After the time 5 mL of dichloromethane was added to dissolve the solid and added 150 mL ethylacetate and filtered the precipitate, washed with ethylacetate. The filtrate was washed with 1N HCl (2X50 mL), 5% $KHSO_4$ (50 mL), dried over sodiumsulfate and concentrated under vacuum. The crude mixture was subjected to silicagel chromatography to afford 650 mg (77%) of pure I-PEG_3-ONH(Boc) **11.1** as an oil; ^{1}H NMR ($CDCl_3$) δ 7.57 (br s, 1H), 4.06-4.0 (m, 2H), 3.80-3.70 (m, 4H), 3.69-3.61(m, 4H), 3.27 (t, *J* = 7 Hz, 2H), 1.48 (s, 9H).

Step 3. To a solution of **11.1** (200 mg, 0.53 mmol) in acetonitrile (6 mL) at room temperature was added $(Boc)_2O$ (175 mg, 0.79 mmol). The reaction mixture was cooled to 0 ^{o}C, added 65 mg (0.53 mmol) of DMAP and stirred at room temperature for 24 h by monitoring the starting material consumption using TLC. Added 20 mL of water to quench the reaction followed by 50 mL saturated $NaHCO_3$ solution. Extracted with 300 mL of ethylacetate and washed with 1N HCl (50 mL), 5% $KHSO_4$ (50 mL), and finally with 50 mL of brine solution. The organic layer was concentrated under vacuum and purified by column chromatography. Yield: 200 mg (79%). ^{1}H NMR ($CDCl_3$) δ 4.10 (t, *J* = 4.5 Hz, 2H), 3.75 (t, *J* = 6 Hz, 4H), 3.71-3.65 (m, 4H), 3.26 (t, *J* = 7 Hz, 2H), 1.54 (s, 18H); ^{13}C NMR ($CDCl_3$) δ 150.0, 83.7, 75.4, 72.0, 70.5, 70.1, 68.6, 28.0, 2.8.

5. Solution phase synthesis and hydrolysis of cypate-acetal 15 (unoptimized)

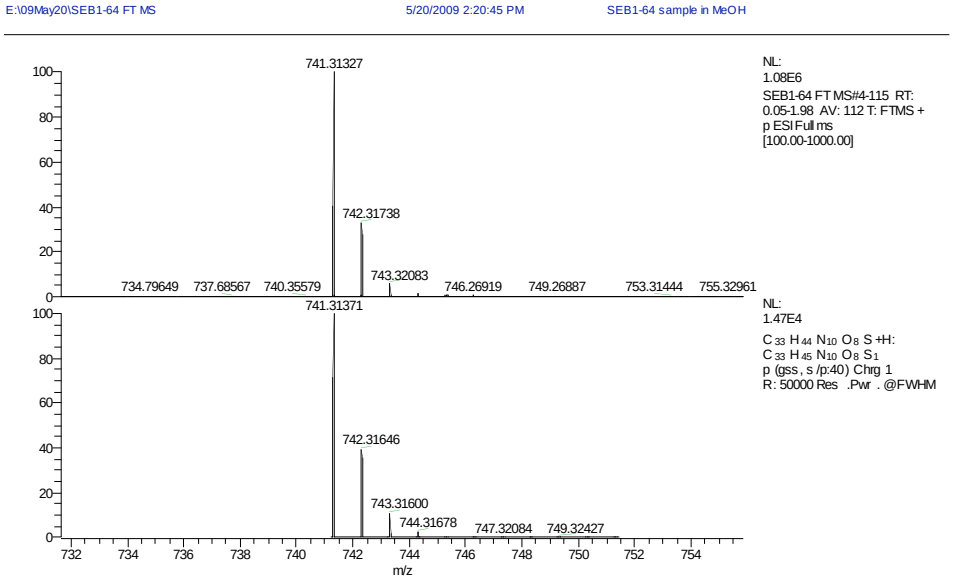
To a suspension of amino acetal $(EtO)_2CH(CH_2)_3NH_2$ (0.010 mL, 0.056 mmol), HOBt (7 mg, 0.052 mmol) and cypate (70 mg, 0.112 mmol) in CH_2Cl_2 (3 mL) at room temperature was added DMF (0.2 mL). The reaction mixture was cooled to 0 °C and DIPEA (0.010 mL, 0.17 mmol) was added followed by addition of a solution of EDC (10 mg, 0.056 mmol) in a 3:2 mixture of CH_2Cl_2:DMF (0.5 mL). After stirring in the dark at room temperature overnight, the reaction was quenched by addition of water and extracted using CH_2Cl_2. The organic layer was washed with brine and then dried (Na_2SO_4). The solvents were removed by rotary evaporation and the residue purified by column chromatography (SiO_2, eluting with 7% MeOH in CH_2Cl_2) to obtain bis-coupled product **15** (7 mg); HRMS, $C_{57}H_{75}N_4O_6^+$ [M + H^+], calcd 911.5681, found 911.5665 (Appendix, Section B5).

*Hydrolysis of **15**.* The bis-acetal was hydrolyzed by dissolving **15** (4.7 mg) in 30% aqueous acetic acid (3 mL) followed by stirring 3.5 h at room temperature. The solvents were then removed under vacuum to obtain aldehyde **16** (3.5 mg) as a green solid; HRMS, $C_{49}H_{55}N_4O_4^+$ [M + H^+], calcd 763.4218, found 763.4227 (Appendix, Section B6).

B. HRMS (FTCIR) Analyses — Spectra of Key Compounds

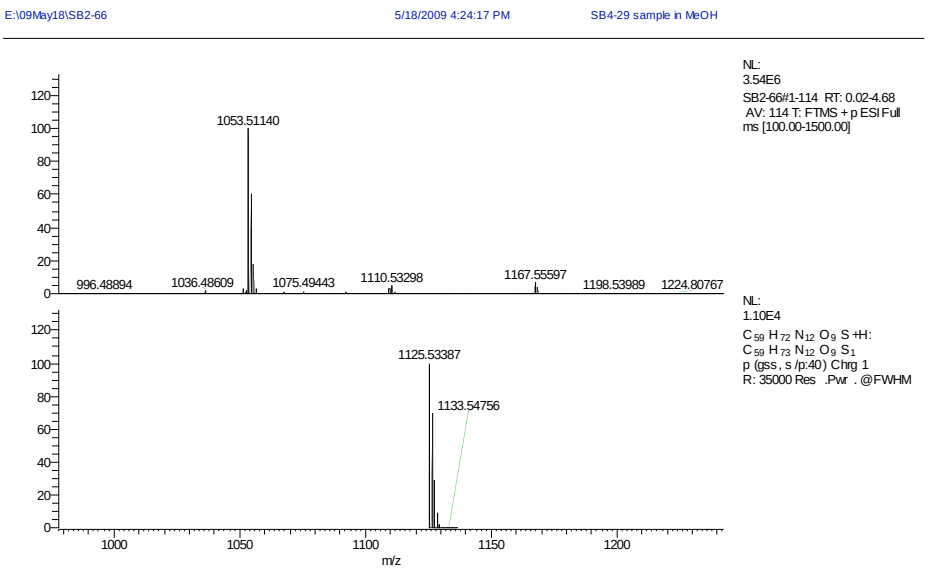
1. Cysteamine hexapeptide conjugate: $HSCH_2CH_2NH–G–G–R–G–G–G\text{-}NHFmoc$
$C_{33}H_{45}N_{10}O_8S$ [M+H$^+$], calcd 741.3137, found 741.3133

top = found, bottom = theoretical

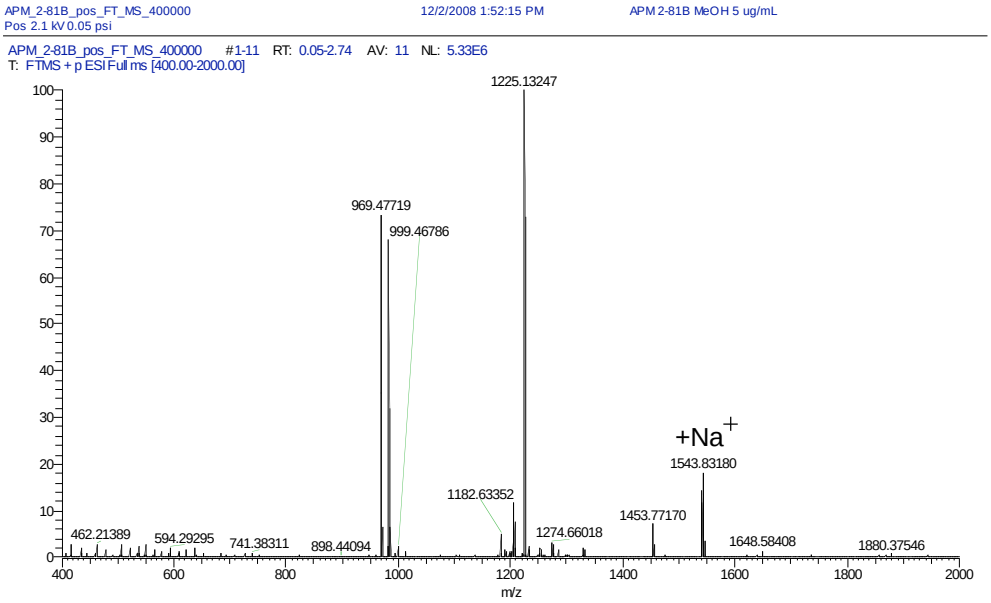


2. Hexapeptide-cypate conjugate: $HSCH_2CH_2NH–G–G–R–G–G–G\text{-}NH\text{-}cypate\text{-}CO_2H$
$C_{56}H_{69}N_{12}O_7S$ [M$^+$– $HO_2CCH{=}CH_2$], calcd 1053.5127, found 1053.5114

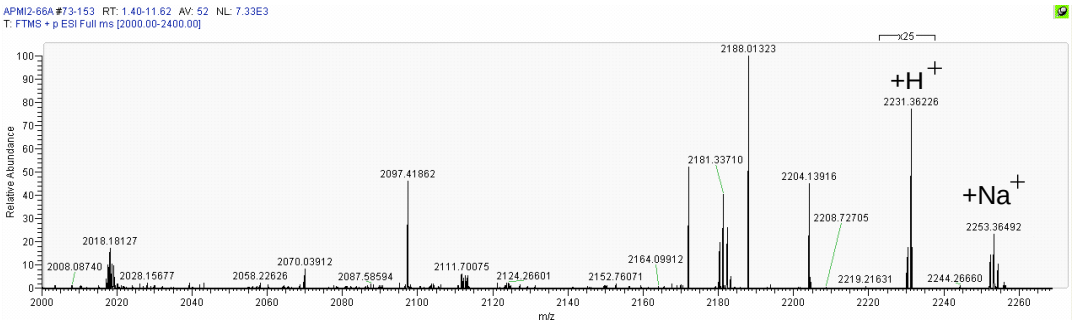
top = found (fragment on loss of acrylic acid), bottom = theoretical (parent)



3. Trityl-cysteamine–PEG$_{11}$–cypate-CO$_2$H (6b):
$C_{89}H_{113}N_4NaO_{16}S$ [M+Na$^+$], calcd 1548.7770, found 1543.8318
$C_{86}H_{109}N_4O_{14}S$ [M+ – H_2C=$CHCO_2H$], calcd 1453.7661, found 1453.7717

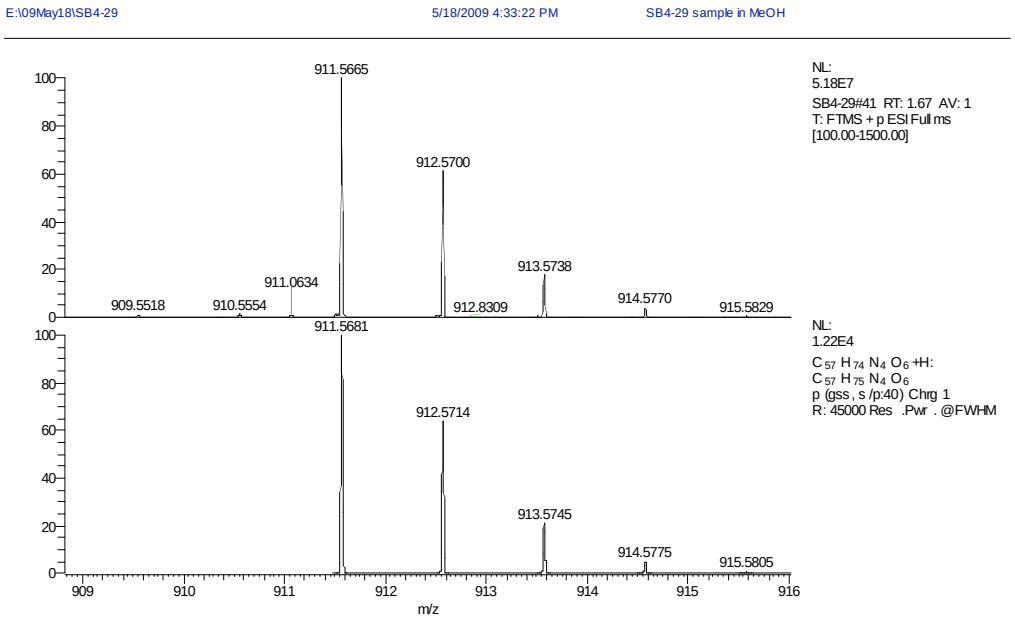


4. Trityl-cysteamine–PEG$_{27}$–cypate-CO$_2$H (6a):
$C_{121}H_{178}N_4O_{32}S$ [M+H$^+$], calcd 2231.2145, found 2231.3623



5. Cypate Bis-Acetal 15: $(EtO)_2HC\text{-}(CH_2)_3\text{-}NH(O)C\text{-cypate-}C(O)NH\text{-}(CH_2)_3\text{-}CH(OEt)_2$
$C_{57}H_{75}N_4O_6^+$ [M + H$^+$], calcd 911.5681, found 911.5665

top = found; bottom = theoretical



6. Cypate Bis-Aldehyde 16: $OHC\text{-}(CH_2)_3\text{-}NH(O)C\text{-cypate-}C(O)NH\text{-}(CH_2)_3\text{-}CHO$
$C_{49}H_{55}N_4O_4^+$ [M + H$^+$], calcd 763.4218, found 763.4227

top = found; bottom = theoretical